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Inhibitory Analogues of the Luteinizing Hormone-Releasing Hormone Having D-Aromatic Residues in Positions 2 and 6 and Variation in Position 3[1]

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A single sc injection of 750 μg/rat of [D-Phe^2,Pro^3,D-Phe^6]-LH-RH on proestrus completely inhibited ovulation in 4-day cycling rats. Ovulation was inhibited partially at 375 μg/rat. At the 750 μg/rat dosage, analogues of LH-RH with D-Phe in positions 2 and 6, and with Sar, Arg, or Hyp in position 3, resulted in partial inhibitors and an inactive analogue with Thr substitution. In the same assay, analogues with D-Phe in position 2 and D-Trp in position 6 were partial inhibitors when D-Phe, Met, or Val were in position 3 and inactive with Ile substitution.

Since our report that a disubstituted LH-RH analogue, [Leu^2,Leu^3]-LH-RH, could inhibit the action of LH-RH in an in vitro assay using isolated whole rat pituitaries,[2] we have synthesized more analogues based on this sequence.[3,4] Of special interest was the design of an LH-RH inhibiting peptide that could effectively suppress ovulation.

The observations by Corbin and co-workers,[5] that [D-Phe^2,D-Ala^6]-LH-RH could inhibit ovulation in rats and rabbits and prevent pregnancy when appropriately administered, precoitally, at a dosage of 24 mg/kg sc, have stimulated the synthesis of analogues of LH-RH having a D-Phe residue in position 2. It was also realized that the incorporation of a D-amino acid residue in position 6, which followed from some of the work of Monahan et al.,[6] was seemingly essential for high antiovulatory activity in rats. However, when the modification of Fujino et al.[7] was applied to the [D-Phe^2,D-Ala^6]-LH-RH sequence, the resulting des-Gly^{10}-[D-Phe^2,D-Ala^6]-LH-RH ethylamide was found to be a superior inhibitor of LH-RH action, in vitro, using a monolayer technique but did not inhibit ovulation.[8]

Recently, Humphries et al.[9] reported that [D-Phe^2,-Pro^3,D-Trp^6]-LH-RH inhibited the release of LH and FSH by LH-RH, in vitro, at a ratio of analogue:LH-RH of 50:1. This analogue also completely inhibited ovulation in 4-day cycling rats at a single sc injection of 750 μg, and partial inhibition of ovulation was also observed at a 375-μg dosage. The analogues, [D-Phe^2,Leu^3,D-Trp^6]-LH-RH and [D-Phe^2,Leu^3,D-Phe^6]-LH-RH were less effective. Bowers and Folkers[10] have shown that the infusion into rats of [D-Phe^2,Pro^3,D-Trp^6]-LH-RH, from a sc implanted, osmotically driven minipump, at a rate of 375 μg/day for 4 days, completely inhibited ovulation in 4-day cycling rats.

This paper describes the synthesis and activities of some further analogues of LH-RH, having a D-Phe residue in position 2, amino acid substitution in position 3, and a D-aromatic amino acid in position 6, in a continuing effort to evaluate structure–activity relationships for inhibition of ovulation.

Experimental Section

The procedure of solid-phase peptide synthesis was essentially identical with that described.[4] Amino acid derivatives were supplied by Peninsula Laboratories, Beckman Bioproducts Division, or Sigma. Product yields (%) were estimated from the starting amino acid-resin. On chromatography, the product of the major peaks was examined by TLC. Usually only those fractions corresponding to the upper parts of the peak were taken, and consequently the percentage yields will be low. Completed, protected peptide-BHA resins were cleaved and deblocked by reaction for 1 h at 0 °C with CoF_3-dried liquid HF[11] containing ca. 20% anisole.

TLC on silica gel was used to evaluate product purity, with the systems R_f^1, EtOAc–H_2O–AcOH–1-BuOH (1:1:1:1 v/v); R_f^2, EtOAc–pyridine–AcOH–H_2O (5:5:1:3 v/v); R_f^3, propan-2-ol–1 N AcOH (2:1 v/v); and R_f^4, 0.1% AcOH–1-BuOH–pyridine (11:5:3 v/v, upper phase). Peptide spots were negative to ninhydrin and positive to chlorine–*o*-tolidine reagent. Amino acid analyses, on ca. 0.5-mg samples hydrolyzed in 6 N HCl in evacuated and sealed ampules for 18 h at 110 °C, were performed using a single column methodology on a Beckman Model 119 amino acid analyzer equipped with an Infotronics Model CRS-210 automatic digital integrator. Optical rotations were measured on a Perkin-Elmer 141 digital read-out polarimeter.

Synthesis of the 3-Substituted [D-Phe^2,D-Phe^6]-LH-RH Analogues. Benzhydrylamine (BHA)-resin hydrochloride (10 g, 0.44 mequiv/g, Beckman Bioproducts) was sized in CH_2Cl_2, neutralized, and coupled with Boc-Gly. The Boc-Gly-BHA resin was submitted to six cycles of deprotection, neutralization, and coupling to yield the heptapeptide BHA-resin, Boc-Ser(Bzl)-Tyr(BrZ)-D-Phe-Leu-Arg(Tos)-Pro-Gly-BHA resin (15.38 g).

Two-gram portions of the heptapeptide BHA-resin were submitted to further coupling cycles, with the appropriate position 3 amino acid derivative, then Boc-D-Phe, and finally Z-<Glu-OH. The protected decapeptide BHA-resins were simultaneously cleaved from the resin and deblocked with HF and purified over Sephadex G-25 (95 × 2.5 cm) with 10% AcOH, followed by ion-exchange chromatography on CM-Sephadex (26 × 1.5 cm) with an NH_4OAc gradient (1 mM, pH 4.5, to 125 mM). In the case of the Arg^3 analogue, 250 mM NH_4OAc was required to elute this highly basic peptide.

[D-Phe^2,Pro^3,D-Phe^6]-LH-RH: yield 423 mg (62%); amino acid analysis gave Glu 1.05, Phe 2 × 1.06, Pro 2 × 1.03, Ser 0.83, Tyr 0.94, Leu 0.91, Arg 0.98, Gly 0.93; R_f^1 0.80, R_f^2 0.91, R_f^3 0.87; $[\alpha]^{24}_D$ −71.60° (*c* 9.38, MeOH).

[D-Phe^2,Sar^3,D-Phe^6]-LH-RH: yield 348.5 mg (52.25%); amino acid analysis gave Glu 1.1, Phe 2 × 1.09, Ser 0.83, Tyr 0.86, Leu 0.97, Arg 1.01, Gly 0.92; R_f^1 0.71, R_f^2 0.80, R_f^3 0.76; $[\alpha]^{24}_D$ −63.59° (*c* 10.3, MeOH).

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Table I. In Vitro Agonist and Antagonist Activity of the Analogues[a]

Peptide analogue	Dose: Peptide, μg/mL of medium	Dose: LH-RH, ng/mL of medium	LH: Δ, ng/mL of medium	LH: SEM (±)	LH: p	FSH: Δ, ng/mL of medium	FSH: SEM (±)	FSH: p
[D-Phe²,Pro³,D-Phe⁶]-LH-RH		0.6	313	62		4855	156	
	0.1	0.6	46	2	~0.001	1375	631	<0.001
	1	0.6	17	4	<0.001	280	513	<0.001
	100		12	5	ns	179	97	ns
			12	2		365	114	
[D-Phe²,Sar³,D-Phe⁶]-LH-RH		0.6	290	48		4372	200	
	0.1	0.6	339	36	ns	4586	428	ns
	1	0.6	127	24	~0.01	3450	434	ns
	10	0.6	22	3	<0.001	518	174	<0.001
	100		13	2	<0.02	321	126	ns
			0.7	3		−75	169	
[D-Phe²,Hyp³,D-Phe⁶]LH-RH		0.6	266	76		4062	192	
	0.1	0.6	179	40	ns	3324	681	ns
	1	0.6	23	3	<0.01	1487	201	<0.001
	10	0.6	15	5	<0.01	−140	333	<0.001
	100		−0.3	1	ns	203	164	ns
			5	2		71	82	
[D-Phe²,Thr³,D-Phe⁶]-LH-RH		0.6	193	18		5421	561	
	0.1	0.6	159	11	ns	5469	561	ns
	1	0.6	97	29	<0.02	2446	811	~0.01
	10	0.6	18	5	0.001	751	278	<0.001
	100		16	3	ns	379	165	ns
			8	7		398	123	
[D-Phe²,Arg³,D-Phe⁶]-LH-RH		0.6	313	62		4855	156	
	0.1	0.6	276	65	ns	5391	409	ns
	1	0.6	51	7	<0.01	1387	229	<0.001
	10	0.6	34	12	~0.001	380	524	<0.001
	100		18	3	ns	−133	151	<0.05
			12	2		365	114	
[D-Phe²,D-Phe³,D-Trp⁶]-LH-RH		0.6	228	30		7469	485	
	0.1	0.6	97	19	~0.01	2118	365	
	1	0.6	39	20	<0.001	70	403	<0.001
	100		10	7	ns	−814	200	ns
			29	13		−757	156	
[D-Phe²,Ile³,D-Trp⁶]-LH-RH		0.6	440	24		10688	1160	
	0.1	0.6	143	24	<0.001	2313	781	<0.001
	1	0.6	21	14	<0.001	−348	158	<0.001
	100		68	21	<0.02	645	195	<0.01
			−1	7		−516	179	
[D-Phe²,Met³,D-Trp⁶]-LH-RH		0.6	408	27		10266	335	
	0.1	0.6	77	12	<0.001	3268	744	<0.001
	1	0.6	52	6	<0.001	−291	476	<0.001
	100		10	6	ns	−672	71	<0.01
			−3	5		−351	55	
[D-Phe²,Val³,D-Trp⁶]-LH-RH		0.6	207	34		10213	896	
	0.1	0.6	44	5	<0.001	3012	535	<0.001
	1	0.6	−32	24	<0.001	−1136	394	<0.001
	100		7	5	ns	110	296	ns
			15	15		142	227	
[D-Phe²,Pro³,D-Trp⁶]-LH-RH[b]		0.6	220	35		6617	601	
	0.03	0.6	26	7	<0.001	−161	334	<0.001
	1	0.6	61	11	<0.01	1270	248	<0.001
	100		9	3	<0.001	175	181	ns
			−15	3		296	106	

[a] For brevity, not all dosages have been reported. [b] See ref 9.

[D-Phe²,Hyp³,D-Phe⁶]-LH-RH: yield 452 mg (65.4%); amino acid analysis gave Glu 1.03, Phe 2 × 0.92, Ser 0.84, Tyr 0.97, Leu 0.90, Arg 0.96, Pro 1.0, Gly 0.91; R_f^1 0.70, R_f^2 0.82, R_f^3 0.74; $[\alpha]^{24}_D$ −52.05° (c 11.2, MeOH).

[D-Phe²,Thr³,D-Phe⁶]-LH-RH: yield 160 mg (23.4%); amino acid analysis gave Glu 0.92, Phe 2 × 0.90, Thr 0.94, Ser 0.84, Tyr 0.99, Leu 1.03, Arg 1.02, Pro 1.02, Gly 0.98; R_f^1 0.83, R_f^3 0.88; $[\alpha]^{24}_D$ −53.94° (c 10.4, MeOH).

[D-Phe²,Arg³,D-Phe⁶]-LH-RH: yield 474.7 mg (66.3%); amino acid analysis gave Glu 0.82, Phe 2 × 1.02, Arg 2 × 1.1, Ser 0.90, Tyr 0.87, Leu 0.87, Pro 1.0, Gly 0.96; R_f^1 0.75, R_f^2 0.87, R_f^3 0.75; $[\alpha]^{24}_D$ −55.35° (c 9.9, MeOH).

Synthesis of the 3-Substituted [D-Phe²,D-Trp⁶]-LH-RH Analogues. The heptapeptide BHA-resin, Boc-Ser(Bzl)-Tyr-(BrZ)-D-Trp-Leu-Arg(Tos)-Pro-Gly-BHA-resin (8.08 g), was synthesized from Boc-Gly-BHA resin (6 g).

Portions of this resin (2 g) were submitted to three further coupling cycles, as before, and the resulting peptide-resins were treated with HF to give the free decapeptides, which were purified over columns of Sephadex G-15 (95 × 2.5 cm) with 33% AcOH and CM-Sephadex.

[D-Phe²,D-Phe³,D-Trp⁶]-LH-RH: yield 180 mg (20.7%); amino acid analysis gave Glu 1.03, Phe 2 × 1.1, Ser 0.78, Tyr 1.05, Leu 0.93, Arg 0.97, Pro 0.99, Gly 0.96; R_f^1 0.78, R_f^3 0.86, R_f^4 0.77; $[\alpha]^{24}_D$ −50.51° (c 9.8, MeOH).

[D-Phe²,Ile³,D-Trp⁶]-LH-RH: yield 250 mg (29.6%); amino acid analysis gave Glu 0.97, Phe 0.89, Ile 0.90, Ser 0.94, Tyr 1.1, Leu 0.97, Arg 1.06, Pro 1.01, Gly 1.01; R_f^1 0.83, R_f^3 0.88, R_f^4 0.77; $[\alpha]^{24}_D$ −42.94° (c 4.05, MeOH).

[D-Phe²,Met³,D-Trp⁶]-LH-RH: yield 233 mg (27.2%); amino

Table II. Antiovulatory Activity of the Analogues

Analogue	Single injection dose (sc), μg/rat	No. of rats	No. of rats ovulated	No. of ova per ovulating rat	SEM (±)	% inhibn of ovulation
[D-Phe^2,Pro^3,D-Phe^6]-LH-RH	375	5	4	8.8	2.4	20
	750	5	0	0	0	100
		5	5	13.2	0.7	0
[D-Phe^2,Sar^3,D-Phe^6]-LH-RH	750	6	3	4	2	50
		4	4	12.5	0.64	0
[D-Phe^2,Hyp^3,D-Phe^6]-LH-RH	750	7	5	7.85	2.1	28
		9	9	13.5	0.53	0
[D-Phe^2,Thr^3,D-Phe^6]-LH-RH	750	4	4	13.2	0.6	0
		5	5	14.4	0.6	0
[D-Phe^2,Arg^3,D-Phe^6]-LH-RH	750	5	3	7.60	3.1	40
		4	4	14.4	0.65	0
[D-Phe^2,D-Phe^3,D-Trp^6]-LH-RH	750	5	3	6.4	2.8	40
		4	4	12.5	1.2	0
[D-Phe^2,Ile^3,D-Trp^6]-LH-RH	750	5	5	12.8	0.6	0
		3	3	11.3	0.3	0
[D-Phe^2,Met^3,D-Trp^6]-LH-RH	750	5	4	10.8	2.8	20
		3	3	11.3	0.3	0
[D-Phe^2,Val^3,D-Trp^6]-LH-RH	1500	5	2	3.2	1.96	60
	750	6	5	9.3	2.5	16
		6	6	13.2	0.5	0
[D-Phe^2,Pro^3,D-Trp^6]-LH-RH[a]		11	11	12.4	1	
	375	9	4	5	3	50
	750	11	0	0	0	100

[a] See ref 9.

acid analysis gave Glu 1.06, Phe 1.07, Met 0.95, Ser 0.84, Tyr 1.08, Leu 0.94, Arg 1.02, Pro 1.09, Gly 0.96; R_f^1 0.77, R_f^3 0.86, R_f^4 0.76; $[\alpha]^{24}_D$ −57.84° (c 11.1, MeOH).

[D-Phe^2,Val^3,D-Trp^6]-LH-RH. This was synthesized in 35% yield from a portion of a different batch of the heptapeptide BHA-resin. Amino acid analysis gave Glu 1.14, Phe 0.89, Val 0.90, Ser 1.03, Tyr 1.04, Leu 0.89, Arg 1.10, Pro 1.03, Gly 1.01; R_f^1 0.58, R_f^2 0.90, R_f^3 0.73; $[\alpha]^{24}_D$ −59.42° (c 9.104, MeOH).

Biological Assays. In vitro studies were performed using pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). The LH and FSH agonist and antagonist activities were determined by incubating for a total of 6 h two pituitaries at 37 °C in 1 mL of lactated Ringer's solution (Abbott Laboratories) in 10-mL teflon beakers in a Dubnoff shaker. Medium was removed each hour for RIA of LH[12] and FSH and fresh medium was added. The LH-RH analogue was added to the incubation medium at I_3, I_4, I_5, and I_6, and LH-RH was added (5 min after the peptide) at I_5 and I_6. Values were calculated in terms of nonograms of the standards LH-LER-1240-2 (0.60 NIH-LH-S1 unit/mg) and NIAMDD rat FSH RP-1 (2.1 × NIH-FSH-S1 units/mg).

Antiovulation assays were performed by counting, on estrus, the number of ova shed by 4-day cycling rats after a single sc injection of the LH-RH analogue in corn oil was administered between 12 and 12:30 p.m. on proestrus. The control rats received 0.3 mL of the vehicle.

Results and Discussion

The in vitro assay data are given in Table I. The analogues, as desired, inhibited LH-RH stimulated gonadotropin release and were essentially devoid of agonist activity at the highest dosages tested.

Of the D-Phe^6 analogues, [D-Phe^2,Pro^3,D-Phe^6]-LH-RH had the highest in vitro potency of inhibition. The inhibition of LH release was essentially complete at 0.1 μg/mL of medium. When Hyp or Arg were in position 3 complete inhibition of LH release was obtained at 1 μg/mL of medium. FSH release appeared to be inhibited less effectively for these Pro, Hyp, and Arg analogues. With Sar or Thr in position 3, partial inhibition of LH and FSH resulted at 1 μg/mL of medium, with the inhibition being complete at 10 μg/mL of medium.

In the case of the D-Trp^6-analogues, practically complete inhibition of LH release at 0.1 μg/mL of medium and complete inhibition of FSH release at 1 μg/mL of medium were observed when Met and Val were in position 3. For the analogues with Ile or D-Phe in position 3, partial inhibition at 0.1 μg/mL of medium and complete inhibition at 1 μg/mL were determined for LH and FSH release.

The rat antiovulatory data are given in Table II. Ovulation was completely inhibited following a single sc injection of 750 μg/rat of [D-Phe^2,Pro^3,D-Phe^6]-LH-RH. At this dosage it was as active as [D-Phe^2,Pro^3,D-Trp^6]-LH-RH.[9] At 375 μg/rat ovulation was partially inhibited. The D-Phe^6-analogues having Sar, Arg, or Hyp residues in position 3 were less effective than the Pro^3 analogue; the Thr analogue was inactive.

The D-Trp^6 analogues having D-Phe, Met, or Val residues in position 3 partially inhibited ovulation at 750 μg/rat; the Ile analogue was inactive.

Analogues, such as [D-Phe^2,Pro^3,D-Phe^6]-LH-RH, that have good antiovulatory activity (complete suppression of ovulation at 750 μg/rat) also have high in vitro potency (complete inhibition at 0.1 μg/mL of medium). However, the converse is not necessarily true since exceptions are known. For example, the related analogue, [cyclopentanecarboxylic acid^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH, inhibits in vitro with about the same high potency as [D-Phe^2,Pro^3,D-Phe^6]-LH-RH but does not suppress ovulation at 750 μg/rat.[13] Good antiovulatory activity of an LH-RH inhibitor, therefore, is not necessarily based on high in vitro potency of inhibition.

The presence of Pro in position 3 of these analogues appears important to both the in vitro and antiovulatory potency. The Pro residue consists of a five-membered, hydrophobic ring in which the α-nitrogen is alkylated. This cyclic residue could influence the orientation of the important first two residues (<Glu^1 and D-Phe^2) and increase potency by strengthening the binding between the inhibitor and receptors (s) or by reducing enzymatic degradation thereby increasing $t_{1/2}$ over that for endogenous LH-RH.

Substitution of more polar residues, such as Hyp, Arg, and Thr, into position 3 led to a decrease in antiovulatory potency. Other substitutions, i.e., Met, D-Phe, Ile, and Val, were not advantageous. However, it was interesting that with Sar in position 3, a 50% inhibition of ovulation was

obtained at 750 μg/rat. The Sar residue consists of a Gly residue in which the α-NH is methylated; it lacks an asymmetric α-carbon. Alkylation of the α-NH moiety of the residue in position 3 may be one factor contributing to the potency of inhibition of ovulation.

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Synthesis of Heteroaromatic Potential β-Adrenergic Antagonists by the Glycidol Route[1]

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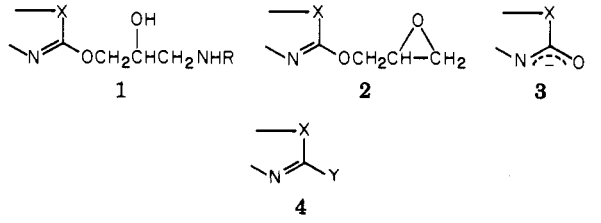
The synthesis of several 3-alkylamino-2-hydroxypropyl heteroaryl ethers (**13–15**, **17**, and **18**) is described. These compounds were prepared by the alkylamination of the corresponding glycidyl ethers (**6–8**, **10**, and **11**), which in turn were obtained from the requisite heteroaryl halides and the sodium salt of glycidol. The above basic ethers exhibited β-blocking activity, but the potency of the tested compounds was considerably less than that of propanolol. Only 3-*tert*-butylamino-2-hydroxyl-1-(1,2,4-thiadiazol-5-yl) propyl ether (**13**) showed some selective myocardial β-blocking activity.

Aromatic compounds in which are incorporated the 1-alkylamino-2-hydroxypropoxy moiety are frequently found to elicit β-adrenergic antagonist activity in vivo.[2] In view of the known[3] myocardial selectivity of 1-alkylamino-3-(2-thiazolyloxy)-2-propanols, it became of interest to determine if this feature, particularly selective myocardial β-blockade, could be found in other heteroaromatic ethers of type **1** where X = O, S, NC_6H_5, or =CH and R = isopropyl or *tert*-butyl.

Chemistry. The synthesis of compounds of type **1** is usually accomplished by the alkylamination of the corresponding glycidyl ethers **2**, which in turn can be obtained from a salt of the oxo compound **3** and an epihalohydrin, provided that alkylation of this ambident anionic system occurs on oxygen.[4] In the event that alkylation of **3** takes place on nitrogen (as is certainly the case for thiazolin-2-ones,[5] 1,2,4-oxadiazolin-5-ones,[6] 1,2,4-thiadiazolin-5-ones,[7] and 1-substituted tetrazol-5-ones[7]), then it is possible to prepare the glycidyl ether **2** via a four-step synthesis from **4** (Y = Cl, Br, etc.) and a salt of glycerol acetonide.[3] It has now been found that the glycidyl ethers **2** can be prepared *in one step* from **4** and glycidol, provided that the nucleophilic displacement of Y occurs easily and that conditions are followed which avoid or minimize the base-induced destruction[8] of glycidol. For example, a solution of the compound to be alkylated (see Table I) and glycidol, in a suitable solvent, was added *slowly* to a suspension of sodium hydride in the same solvent. The crucial aspect of this process is that the concentration of the sodium salt of glycidol is maintained at a low level and thus the self-polymerization thereof is largely averted. The yields of the glycidyl ethers were, in general, satifactory, although on occasion the isolation of a pure product from the reaction mixture was difficult. In those cases (compounds **5–7**, Table I) it was expedient to convert the crude epoxide directly into the desired amino compound (see Table II). For the examples cited herein, the overall yields of the amino alcohols (Table II) were equivalent to, or better than, those obtained by the glycerol acetonide route.



The reaction of *tert*-butylamine with the glycidyl ether **9** gave 3-phenyl-5-*tert*-butylamino-1,2,4-oxadiazole (**16**) instead of the required amino alcohol. A similar dis-

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